Biochem. J. (1986) 239, 777–780 (Printed in Great Britain)

Determination of the molecular mass of apolipoprotein B-100

A chemical approach

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Apolipoprotein B-100 (apo B-100) is the protein ligand in low-density lipoproteins that binds to a specific cell-surface receptor. Its molecular mass has been a subject of controversy. We have determined the molecular mass of the protein by a chemical approach. After complete CNBr cleavage, the *C*-terminal fragment of apo B-100 was purified by reverse-phase h.p.l.c. Amino acid *N*- and *C*-terminal analyses confirm that this peptide represents the *C*-terminal peptide as deduced from the DNA sequence of a human apo B-100 cDNA clone. A chemically synthesized peptide was used to determine the recovery of the peptide (74.72%). On the basis of these data, the molecular mass of apo B-100 was determined to be 496.82 ± 24.84 kDa.

INTRODUCTION

The low-density lipoproteins (LDL) of human plasma contain a high-molecular-mass protein, apolipoprotein B (apo B), which plays an important role in lipid transport. It is the ligand that binds to the LDL receptor, thereby regulating cholesterol homoeostasis [1]. Two forms of apo B, namely apo B-100 and apo B-48, have been identified in man [2]; apo B-100 is synthesized by the liver and is an obligatory constituent of LDL, IDL and VLDL. Apo B-48 is synthesized by the intestine and is found in chylomicrons and chylomicron remnants. Human plasma LDL contains approx. 80% lipids and 20% protein by weight [3,4]. Approx. 4–10% of apo B's mass consists of carbohydrate chains containing galactose, mannose, *N*-acetylglucosamine and sialic acid residues [5–7]. Estimates of the molecular mass of apo B by other investigators have ranged from 8000 to 550000 Da [2,8–11].

Structural analysis of apo B-100 has been greatly hampered by its insolubility in aqueous buffers after delipidation, although its amino acid composition [2,4] is not particularly dominated by hydrophobic amino acids. In our laboratory, the application of h.p.l.c. techniques had made it possible to isolate pure tryptic-digest and CNBr-cleavage peptides of apo B.

In the present study we report the determination of the molecular mass of apo B by a novel chemical approach. We have performed a CNBr cleavage of intact apo B-100 and have quantified the exact molar amount of the *C*-terminal peptide released. The yield of the peptide on the h.p.l.c. was monitored by control experiments with a synthetic peptide.

EXPERIMENTAL

Isolation of LDL and apo B preparation

Human plasma was obtained by plasmapheresis from normal fasting donors. LDL was isolated according to the standard procedure by ultracentrifugation in KBr solutions [12]. The protein was analysed by electrophoresis on 0.1% SDS/5–14% gradient polyacrylamide slab gels [13] and was shown to contain only one protein band at the position of apo B-100. The molecular mass of the protein was estimated by including a weight standard (Bio-Rad Laboratories) in the gel. Protein concentration was determined by the modified Lowry method of Markwell *et al.* [14], with bovine serum albumin as the standard. The values obtained by this method agreed with those determined by amino acid analysis. LDL was delipidated and freeze-dried [12] before CNBr cleavage.

CNBr cleavage [15] and h.p.l.c. purification of peptides

A 5 mg portion of apo B in 70% (v/v) formic acid was added to a 4-fold or 100-fold weight excess of CNBr. The reaction mixture was sealed under N_2 and maintained for 18 h in the dark at room temperature. The solution was then diluted 3-fold with distilled water and dried in a Speedvac (Savant Instruments Co.). The peptide mixture was reconstituted with 1 ml of 20% formic acid. A 200 μl portion of the mixture was injected on to a Waters h.p.l.c. system. The CNBr-cleaved *C*-terminal (peptide C) was purified on a Vydac C_{18} column (4.6 mm × 250 mm) at 50 °C by using a trifluoroacetic acid buffer system [buffer A, 0.1% trifluoroacetic acid in water; buffer B, 0.08% trifluoroacetic acid in aq. 95% (v/v) acetonitrile] [16]. The gradient ran from 15% (v/v) solvent B to 40% solvent B in 30 min and then from 40% solvent B to 100% solvent B in 2 min.

Peptide synthesis and purification

An Applied Biosystems 430A solid-phase peptide synthesizer was used to synthesize the apo B-100 CNBr-cleavage *C*-terminal fragment. After desalting, purification of the synthetic peptide was carried out according to the rechromatography system of Yang [17].

Amino acid sequencing and analysis

A gas-phase automatic sequencer [18] and a manual sequencing technique [19] were used to sequence the

Abbreviations used: LDL, VLDL and IDL, low-density, very-low-density and intermediate-density lipoproteins respectively; apo B-100, apolipoprotein B-100.

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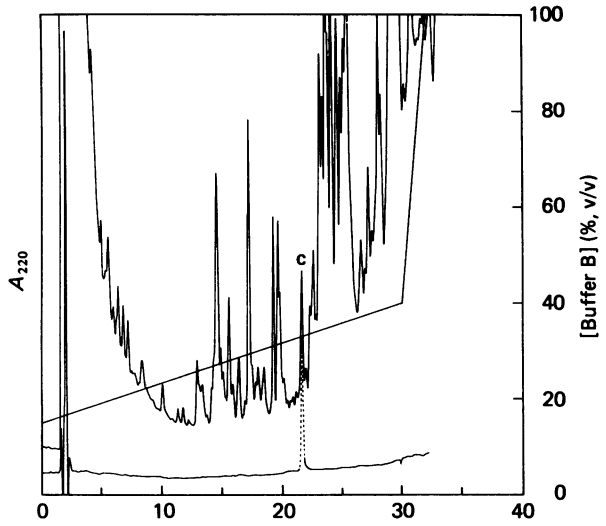

Fig. 1. H.p.l.c. of the apo B-100 CNBr-cleavage peptides on a Waters h.p.l.c. system with the trifluoroacetic acid buffer system and a flow rate of 1.5 ml/min at 50 °C

For experimental conditions see the text. The broken line under peak C is the absorption peak from a separate injection of synthetic peptide C.

C-terminal peptide. Amino acid phenylthiohydantoin derivatives were identified with a Brownlee C_{18} column (4.6 mm × 250 mm) on a Beckman h.p.l.c. system. Amino acid dimethylaminoazobenzenethiohydantoin derivatives were identified by either t.l.c. [19,20] or an h.p.l.c. [21] method. An LKB amino acid analyser using ninhydrin as reagent and a Waters Picotag h.p.l.c. system using phenyl isothiocyanate for pre-column formation of derivatives were used to quantify the amino acid composition. Phenylthiocarbamoyl-amino acids were prepared according to the Waters Picotag procedure with minor modifications [22].

C-Terminal analysis

For analysis of apo B-100 *C*-terminal amino acid residues, the methods using hydrazine [23] and carboxypeptidase were carried out.

Hydrazinolysis. The sample (2 nmol) was sealed in a glass tube with 0.2 ml of hydrazine at 100 °C for 8 h [23,24]. The tube was then opened and the sample was dried by a Speedvac overnight. To avoid charging the analyser column with undue amounts of hydrazides, a modified procedure described by Braunitzer was applied [25]. The samples were analysed by an LKB amino acid analyser.

Carboxypeptidase Y cleavage. The sample (2 nmol) was dissolved in 0.1 M-pyridine/acetate buffer, pH 6.0, and then digested with 100 μl of Pierce immobilized carboxypeptidase Y (10–14 units/ml) at room temperature. The mixture was shaken occasionally. Samples were taken at time points of 2, 6, 30 and 60 min. To control autodigestion by carboxypeptidase Y, the same amount of enzyme was incubated and taken simultaneously. Samples were dried and coupled with phenyl isothiocyanate for phenylthiocarbamoyl-amino acid analysis [22].

Calibration of peptide yield by h.p.l.c. separation

To calibrate the yield of the apo B-100 CNBr-cleavage *C*-terminal peptide after Vydac C_{18} column separation, a similar amount of the synthetic peptide was injected on to the h.p.l.c. system under identical conditions. The concentration of the synthetic peptide C was 0.674 nmol/10 μl. Samples (20 μl, 24 μl and 30 μl) were injected separately on to the column. The peptide was collected and quantified with an LKB amino acid analyser and a Waters Picotag system. Each of the experiments was repeated three times. The yield of the apo B-100 CNBr-cleavage *C*-terminal peptide from h.p.l.c. was expressed as a percentage of the amount of peptide fed into the system.

RESULTS AND DISCUSSION

The actual molecular mass of apo B-100 has been a subject of considerable controversy [2,8–11]. Because of the large size and insolubility of the protein, little sequence information is available on the protein. In this study, we have deduced the molecular mass of apo B-100 by a chemical approach.

H.p.l.c. purification of the apo B-100 CNBr-cleavage fragment under the described conditions offers direct isolation of pure peptide C, which contains no methionine. Therefore the two possible forms of each peptide obtained by CNBr cleavage, i.e. the peptide with homoserine lactone and homoserine, can be ruled out, and the molecular mass of apo B-100 can be estimated.

Separation of apo B-100 CNBr-cleavage fragment and characterization of the *C*-terminal peptide

Fig. 1 shows the separation of the apo B-100 CNBr-cleavage fragment on a Vydac C_{18} column with a Waters h.p.l.c. system using a trifluoroacetic acid buffer system. The fraction under each peak is collected and sequenced by the modified Edman degradation method for three cycles to check its purity. Although the chromatogram pattern is complex, with multiple peaks, many of the peaks consisted of analytically pure

Table 1. Amino acid composition of apo B-100 CNBr-cleavage *C*-terminal fragment (CN-CT) and its synthetic analogue peptide (Synt. CT)

Results for CN-CT and Synt. CT are given as numbers of residues in molar proportions after 1 h hydrolysis with 5.7 M-HCl and 0.0125% phenol at 150 °C. Row A gives the number of residues according to the peptide sequence.

	Glu	Gly	Thr	Ala	Pro	Ile	Leu	Lys	Total
CN-CT	1.24	0.99	0.90	0.98	0.91	1.43	2.92	1.05	10.43
Synt. CT	1.18	1.06	0.91	0.96	0.99	1.53	3.00	0.90	10.53
A	1	1	1	1	1	2	3	1	11

Table 2. Amino acid sequence of apo B-100 CNBr-cleavage *C*-terminal fragment

Key: –, gas-phase sequencing; →, modified Edman degradation; ←, carboxypeptidase Y *C*-terminal analysis; *, hydrazinolysis.

Lys	Leu	Ala	Pro	Gly	Glu	Leu	Thr	Ile	Ile	Leu
–	–	–	–	–	–	–	–	–		
→	→	→	→	→	→	→	→	→		
								←	←	←
										*

peptides. Peak C (Fig. 1) was found to be a pure peptide. Amino acid analysis (Table 1) and *N*-terminal analysis of the first nine residues of peptide C exhibited results identical with the apo B-100 *C*-terminal sequence deduced by nucleotide sequencing of a human apo B-100 cDNA clone [26,27]. The sequence of this peptide is shown in Table 2.

Hydrazine and carboxypeptidase Y were used for *C*-terminal analysis of peptide C. Our results confirmed that leucine is the *C*-terminal amino acid residue of peptide C and apo B-100. Peptide C was eluted at 33% solvent B of the trifluoroacetic acid buffer system and had the same amino acid sequence as that deduced from a human cDNA clone, confirming that it is the CNBr-cleavage *C*-terminal fragment of apo B-100.

Determination of the molecular mass of apo B-100

The direct isolation of the *C*-terminal CNBr-cleavage fragment of apo B-100 in pure form made the determination of the molecular mass of apo B-100 possible, since only one peptide form was released. To confirm that cleavage was complete, a 4-fold and a 100-fold weight excess of CNBr were left to react with apo B-100 in 70% formic acid for 18 h. Our amino acid analysis results revealed that the recovery of peptide C was identical in both instances by injection of the same amount of the peptide mixture (for details see the Experimental section). The average yield of peptide C on the h.p.l.c. is 74.2%. The concentration of the CNBr-cleavage fragment of apo B-100 is 1.095 mg/200 μl. A 200 μl portion of this mixture was injected and peptide C was collected. On the basis of the average of the amino acid analysis data from eight separate experiments, 1.647 ± 0.08 nmol of peptide C is isolated from 1.095 mg of apo B-100. Since the h.p.l.c. yield of peptide C under the experimental conditions is 74.72%, 1.095 mg of apo B-100 should contain 2.204 ± 0.115 nmol of peptide C. According to such analysis, the molecular mass of apo B-100 should be 496.82 ± 24.84 kDa. It should be noted that this determination does not include the carbohydrate attached to the protein. Apo B-100 of LDL is a glycoprotein. The protein amount determined by the Lowry method does not include the sugar on the apo B-100. Carbohydrate represents approx. 4–10% of the total apo B mass as reported by other investigators [5–7]. The actual molecular mass of apo B-100 should thus range from 517 kDa to 547 ± 25 kDa, an estimate that is close to the results as reported by Kane *et al.*, 550 kDa [2]. According to the amino acid composition of apo B-100, the average molecular mass of the 20 common amino acids present in apo B-100 is 112, and apo B-100 can be estimated to contain 4436 ± 222 amino acid residues.

Partial cDNA clones of apo B-100 have been reported by a number of laboratories [26–29]. Presumably the complete amino acid sequence of the protein will ultimately be deduced from the DNA sequence. However, an accurate direct estimation of the molecular mass of apo B-100 is important for a number of reasons. For example, apo B-100 mRNA is reported to be up to 22 kilobases, containing enough information to code for well over 5000–6000 amino acid residues [29]. Our study suggests that apo B-100 contains not more than 4658 residues. The reported apparent molecular mass of apo B-100 mRNA was possibly in error, since DNA instead of RNA markers were used as standards. Alternatively, long stretches of untranslated sequences might be present in the mRNA. One final possible explanation for the apparent discrepancy is that the secreted protein present in LDL has been processed proteolytically. The evidence for or against some of these possibilities should be forthcoming in the near future.

We thank B. Touchstone, B. Beuermann, M. Taffere and T. M. Yang for excellent technical assistance, Mrs. S. Kelly for the artwork and Ms. M. Sampel for preparation of this manuscript. This work was supported by an S.C.O.R. on Arteriosclerosis (HL-27341) and the American Heart Association, Texas Affiliate Grant no. 85G-202.

REFERENCES

1. Brown, M. S. & Goldstein, J. L. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 3330–3337
2. Kane, J. P., Hardman, D. A. & Paulus, H. E. (1980) Proc. Natl. Acad. Sci. U.S.A. **787**, 2465–2469
3. Skipski, V. P. (1972) in Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism (Nelson, G. J., ed.), pp. 471–583, Wiley–Interscience, New York
4. Chapman, M. J., Goldstein, S. & Mills, G. L. (1978) Eur. J. Biochem. **87**, 475–488
5. Scanu, A. M. & Ritter, M. C. (1973) Adv. Clin. Chem. **16**, 111–153
6. Eisenberg, S. & Levy, R. I. (1975) Adv. Lipid Res. **13**, 1–89
7. Swaminathan, N. & Aladjem, F. (1976) Biochemistry **15**, 1516–1522
8. Socorro, L. & Camejo, G. (1979) J. Lipid Res. **20**, 631–638
9. Krishnaiah, K. V. & Weigandt, H. (1974) FEBS Lett. **40**, 265–268
10. Shore, B. (1957) Arch. Biochem. Biophys. **71**, 1–10
11. Cardin, A. D., Witt, K. R., Chao, J., Margolius, H. S., Donaldson, V. H. & Jackson, R. L. (1984) J. Biol. Chem. **259**, 8522–8528
12. Cardin, A. D., Witt, K. R., Barnhart, C. L. & Jackson, R. L. (1982) Biochemistry **21**, 4503–4511
13. Mattick, J. S., Nickless, J., Mizugaki, M., Yang, C. Y., Uchiyama, S. & Wakil, S. J. (1983) J. Biol. Chem. **258**, 15300–15304
14. Markwell, M.-A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) Anal. Biochem. **87**, 206–210
15. Gross, E. (1967) Methods Enzymol. **11**, 238–255
16. Yang, C. Y., Pownalla, H. J. & Gotto, A. M., Jr. (1985) Anal. Biochem. **145**, 67–72
17. Yang, C. Y., Pauly, E., Kratzin, H. & Hilschmann, N. (1981) Hoppe-Seyler's Z. Physiol. Chem. **362**, 1131–1146
18. Hewick, R. M., Hunkapillar, M. W., Hood, L. E. & Dryer, W. J. (1981) J. Biol. Chem. **256**, 7990–8025
19. Chang, J. Y., Brauer, D. & Wittmann-Liebold, B. (1978) FEBS Lett. **93**, 205–214
20. Yang, C. Y. (1979) Hoppe-Seyler's Z. Physiol. Chem. **360**, 1673–1675
21. Yang, C. Y. & Wakil, S. J. (1984) Anal. Biochem. **137**, 54–57

22. Yang, C. Y. & Sepulveda, F. I. (1985) J. Chromatogr. **346**, 413–416
23. Akabori, S., Ohno, K. & Marita, K. (1952) Bull. Chem. Soc. Jpn. **25**, 214–218
24. Fraenkel-Conrat, H. & Tsung, C. M. (1967) Methods Enzymol. **11**, 151–155
25. Braunitzer, G. (1957) Chem. Rev. **88**, 2025–2036
26. Wei, C. F., Chen, S. H., Yang, C. Y., Marcel, Y. L., Milne, R. W., Li, W. S., Sparrow, J. T., Gotto, A. M., Jr. & Chan, L. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7265–7269
27. Knott, T. J., Rall, S. C., Jr., Innerarity, T. L., Jacobson, S. F., Urdea, M. S., Levy-Wilson, B. J., Powell, L. M. Pease, R. J., Eddy, R., Nakai, H., Byers, M., Priestley, L. M., Robertson, E., Rall, L. B., Betsholtz, C., Shows, T. B., Mahley, R. W. & Scott, J. (1985) Science **230**, 37–43
28. Law, S. W., Lackner, K. J., Hospattankar, A. V., Anchors, J. M., Sakaguchi, A. Y., Naylor, S. L. & Brewer, H. B., Jr. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8340–8344
29. Huang, L. S., Bock, S. C., Feinstein, S. I. & Breslow, J. L. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6825–6829

Received 15 July 1986/13 August 1986; accepted 21 August 1986